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TCP Transcription Factors Control the Morphology of Shoot Lateral Organs via Negative Regulation of the Expression of Boundary-Specific Genes in *Arabidopsis* [W][OA]

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Plants form shoot meristems in the so-called boundary region, and these meristems are necessary for normal morphogenesis of aerial parts of plants. However, the molecular mechanisms that regulate the formation of shoot meristems are not fully understood. We report here that expression of a chimeric repressor from TCP3 (TCP3SRDX), a member of TEOSINTE BRANCHED1, CYCLOIDEA, and PCF (TCP) transcription factors in *Arabidopsis thaliana*, resulted in the formation of ectopic shoots on cotyledons and various defects in organ development. Expression of TCP3SRDX induced ectopic expression of boundary-specific genes, namely the *CUP-SHAPED COTYLEDON* (*CUC*) genes, and suppressed the expression of *miR164*, whose product cleaves the transcripts of *CUC* genes. This abnormal phenotype was substantially reversed on the *cuc1* mutant background. By contrast, gain of function of TCP3 suppressed the expression of *CUC* genes and resulted in the fusion of cotyledons and defects in formation of shoots. The pattern of expression of *TCP3* did not overlap with that of the *CUC* genes. In addition, we found that eight TCPs had functions similar to that of TCP3. Our results demonstrate that the TCP transcription factors play a pivotal role in the control of morphogenesis of shoot organs by negatively regulating the expression of boundary-specific genes.

INTRODUCTION

Meristems are composed of small populations of undifferentiated cells. Plants can regenerate entire organs from shoot meristems via the production of organ primordia. Once organ primordia have been generated, they differentiate into various organs according to their specified fates and cannot return to the meristematic phase during normal development. Lateral organ primordia are established in the peripheral region of a shoot meristem, which is associated with the formation of boundaries that separate organ primordia from the shoot meristem (Aida and Tasaka, 2006a, 2006b). In dicotyledonous plants, a shoot apical meristem (SAM) is formed in the boundary region of two cotyledonary primordia during embryogenesis, and secondary shoot meristems are formed at the boundary of stems and leaves, namely, at leaf axils.

Several factors have been identified as regulators of the formation of shoot meristems in boundary regions. The expression of boundary-specific genes for NAC domain transcription factors, namely, *NO APICAL MERISTEM*, *CUP-SHAPED COTYLEDON* (*CUC*), and *CUPULIFORMIS* in petunia (Petunia hybrida), *Arabidposis thaliana*, and *Antirrhinum majus*, respectively, is necessary for the initiation of formation of shoot meristems (Souer et al., 1996; Aida et al., 1997; Vroemen et al., 2003; Weir et al., 2004). Moreover, loss of expression of two of the three *CUC* genes in *Arabidopsis* results in the fusion of cotyledons and defects in the formation of the SAM (Aida et al., 1997; Vroemen et al., 2003). By contrast, ectopic expression of *CUC1* enhances the expression of class I *KNOTTED1*-like homeobox (*KNOX*) genes and induces the formation of ectopic shoots on the adaxial surface of *Arabidopsis* cotyledons (Takada et al., 2001; Hibara et al., 2003). This observation indicates that the expression of the *CUC1* gene is sufficient for the induction of formation of ectopic shoots on cotyledons. The expression of *CUC* genes is detected only at the boundaries of embryonic cotyledonary primordia and postembryonic organs, such as leaf axils and floral organs (Aida et al., 1999; Takada et al., 2001; Vroemen et al., 2003; Hibara et al., 2006). Thus, the spatially restricted expression of *CUC* genes is important for the formation of a shoot meristem at a defined position. Although it has been suggested that the auxin response pathway and microRNA (miRNA) might be involved in the control of the expression of *CUC* genes (Vernoux et al., 2000; Aida et al., 2002; Furutani et al., 2004; Laufs et al., 2004; Mallory et al., 2004; Baker et al., 2005), the molecular mechanisms that regulate the spatial expression of *CUC* genes, which are involved in the formation of shoot meristems, remain unknown.

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The TEOSINTE BRANCHED1, CYCLOIDEA, and PCF (TCP) family of transcription factors has been reported to play roles in various aspects of plant development (Luo et al., 1996; Doebley et al., 1997; Kosugi and Ohashi, 1997; Cubas et al., 1999). Loss of function of a *TCP* gene, the *CINCINNATA* (*CIN*) gene, in *Antirrhinum* results in the abnormal curvature of leaves and petals (Nath et al., 2003; Crawford et al., 2004). In addition, suppression of the expression of *TCP* genes by the ectopic expression of *miR319/JAW*, which targets *TCP2*, *TCP3*, *TCP4*, *TCP10*, and *TCP24* genes, induced a *cin*-like phenotype in *Arabidopsis* (Palatnik et al., 2003). The *Arabidopsis* genome contains 24 *TCP* genes, which have been classified into the CYC/TB and PCF subfamilies (see Supplemental Figure 1 online; Cubas, 2000). However, the functional roles of members of the TCP family, including CIN, remain to be clarified.

We developed a gene silencing system, designated chimeric repressor gene-silencing technology (CRES-T), in which a transcription factor fused to the EAR-motif repression domain (SRDX) dominantly represses the transcription of its target genes, even in the presence of endogenous and functionally redundant transcription factors (Hiratsu et al., 2003). Using the CRES-T system, which involves generation of a dominant repressor, we have obtained some insights into the function of TCPs in the regulation of morphogenesis of shoot lateral organs, including the formation of the shoot meristems, via negative control of the expression of boundary-specific genes.

RESULTS

The Chimeric TCP3 Repressor Induces the Formation of Ectopic Shoots

As part of our efforts to identify the biological functions of TCP transcription factors, we applied our CRES-T system to TCP transcription factors because we observed no visible abnormal phenotypic features in either *Arabidopsis* T-DNA–tagged lines for the *TCP* genes (data not shown). We converted TCP3, which is phylogenetically close to CIN (see Supplemental Figure 1 online), into a chimeric repressor by fusing it with the SRDX repression domain (Hiratsu et al., 2003), and we expressed the fused gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter (*35S:TCP3SRDX*; Figure 1A) in *Arabidopsis*. Expression of *TCP3SRDX* induced various morphological abnormalities, which were specifically evident on the surface and at the margins of various organs. Cotyledons of *35S:TCP3SRDX* plants were wavy, serrated, and much smaller than those of the wild type, and a number of ectopic shoots were generated on the adaxial side of the cotyledons (Figures 1B to 1E). The abnormal phenotypes of *35S:TCP3SRDX* seedlings could be grouped into three classes according to the severity of abnormalities. Seedlings with a mildly abnormal phenotype had epinastic cotyledons with indistinctly differentiated petioles and blades but no ectopic shoots (Figure 1C). Seedlings with a moderate phenotype had cotyledons with serrations at their margins and many ectopic shoots (Figure 1D). The seedlings with a severe phenotype had multiple ectopic shoots on their cotyledons, which resulted in defects in the expansion of the cotyledon (Figures 1E and 2B). In addition, we often observed ectopic trichomes on the surface of the cotyledons (Figure 2B). Severe defects in the elongation of the main root were also frequent (see Supplemental Figure 2 online), and the pattern of vein formation was severely disrupted in the cotyledons of *35S:TCP3SRDX* plants (Figures 1F and 1G). Because the defects in the cotyledons were also evident in mature embryos (Figures 1H and 1I), we postulated that the abnormal development of the cotyledons in *35S:TCP3SRDX* plants occurred during embryogenesis.

The seedlings of *35S:TCP3SRDX* plants with a mild phenotype grew relatively normally, but their rosette leaves were wavy and serrated (Figures 1J and 1K), resembling those of *jaw-D* plants (Palatnik et al., 2003). The *35S:TCP3SRDX* plants with a moderate or severe phenotype often failed to grow when transferred to soil, and those that developed to rosette plants usually had a bushy phenotype, which was probably due to the presence of ectopic shoots and suggested that the ectopic shoots induced by TCP3SRDX had functional meristems (Figures 1L and 1M). Ectopic shoots were occasionally generated, but at low frequency, on the leaves of *35S:TCP3SRDX* plants (data not shown). The morphology of the floral organs of *35S:TCP3SRDX* plants was severely abnormal (Figures 1N to 1U). The sepals and petals were wavy and serrated (Figures 1Q and 1S), and the stamens often had clumped outgrowths of cells on their anthers (Figure 1U). Carpels of *35S:TCP3SRDX* plants appeared normal, but the surface of siliques was crinkled and the siliques were significantly shorter than those of the wild type (Figure 1V). These results indicated that *TCP3SRDX* was able to induce abnormal development in various organs, regardless of their identity.

Observations by scanning electron microscopy revealed that wild-type cells in the epidermis and the marginal region were organized in a specific pavement-like pattern and were rod-shaped, respectively, whereas the cells of *35S:TCP3SRDX* plants were rounded both in the epidermis and in the marginal regions, with features of undifferentiated cell clusters (Figures 2C to 2F; Donnelly et al., 1999; Ori et al., 2000). Because the differentiation of cells is regulated by their relative position within an organ (Donnelly et al., 1999; Ori et al., 2000), these observations suggested that the epidermal cells of *35S:TCP3SRDX* plants do not undergo position-dependent differentiation.

A transient expression assay revealed that TCP3 had transactivation activity and that TCP3SRDX acted as a repressor in *Arabidopsis* leaves (see Supplemental Figure 3 online). In addition, *TCP3mSRDX*, in which the encoded amino acid sequence of the SRDX repression domain was mutated (mSRDX; Hiratsu et al., 2004), had no repressive activity (see Supplemental Figure 3 online). We confirmed that the expression of *35S:TCP3mSRDX* in transgenic *Arabidopsis* was unable to induce morphological defects in cotyledons and in leaves (data not shown). These results indicated that the phenotype of *35S:TCP3SRDX* plants was induced by the repressive activity of TCP3SRDX and not by some nonspecific negative effect(s), such as squelching (Cahill et al., 1994).

We expressed *TCP3SRDX* under the control of the 5′-upstream region of the *TCP3* gene (Pro_{TCP3}*:TCPS3RDX*), instead of the CaMV 35S promoter, to examine the activity of TCP3SRDX in a condition similar to that of native TCP3. We found that Pro_{TCP3}*:TCPS3RDX* plants had the same phenotype as that of *35S:TCP3SRDX* plants, although the frequency of the moderate

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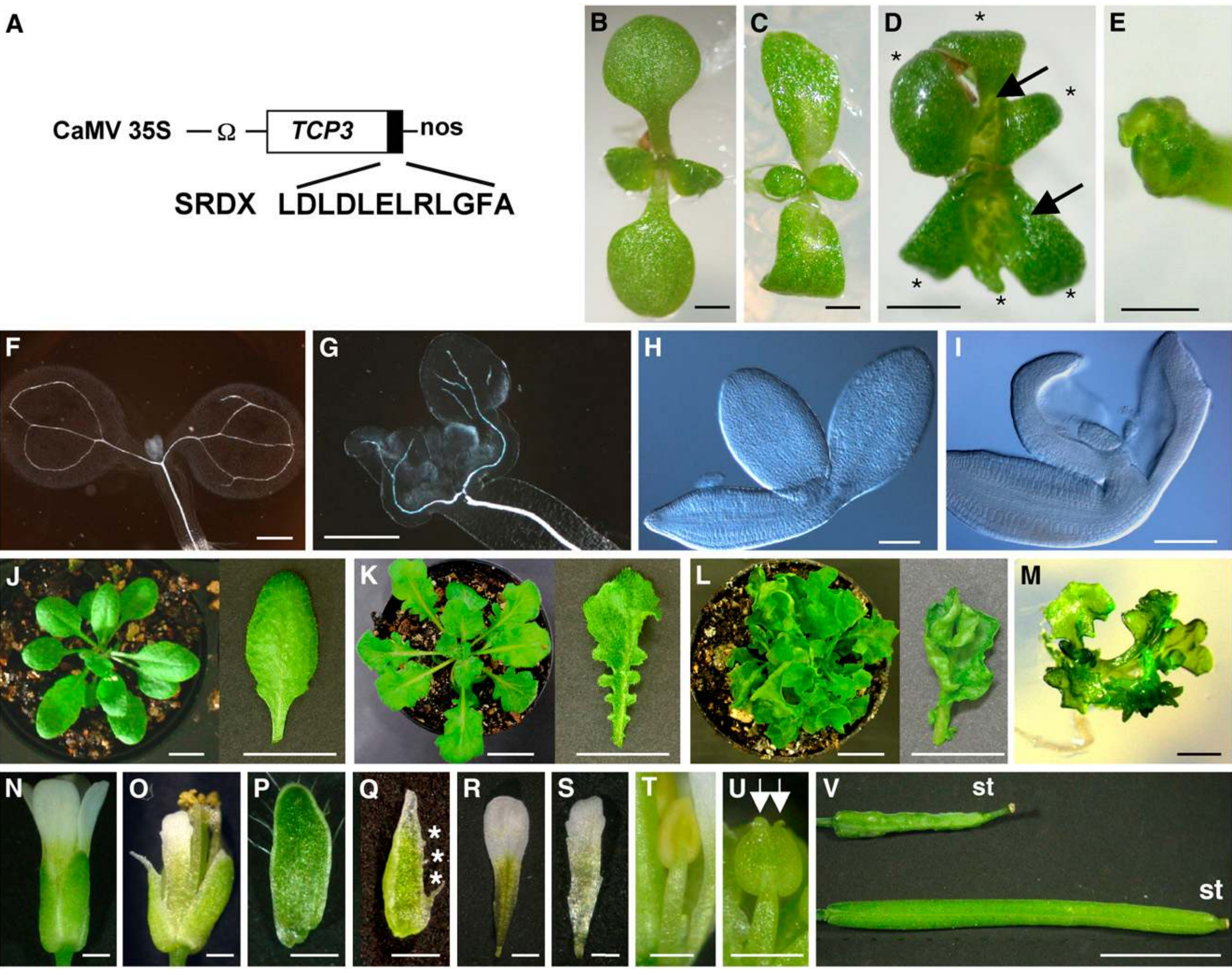

Figure 1. Abnormal Phenotype of Various Organs Induced by TCP3SRDX.

(A) Schematic representation of the *35S:TCP3SRDX* gene. CaMV 35S, Ω, SRDX, and nos represent the CaMV 35S promoter, the translational enhancer of *Tobacco mosaic virus*, the repression domain of 12 amino acids, and the terminator sequence of the *NOS* gene, respectively.
(B) to **(E)** Seedlings of wild-type **(B)** and *35S:TCP3SRDX* plants with the mild **(C)**, moderate **(D)**, and severe **(E)** phenotypes. The asterisks and arrows in **(D)** indicate lobes and ectopic shoots of cotyledons, respectively.
(F) and **(G)** The patterns of vasculature in seedlings of wild-type **(F)** and *35S:TCP3SRDX* **(G)** plants.
(H) and **(I)** Mature embryos of the wild-type **(H)** and the T2 generation of *35S:TCP3SRDX* **(I)** plants.
(J) to **(M)** A rosette and leaf of a wild-type plant **(J)** and of *35S:TCP3SRDX* plants with the mild **(K)**, moderate **(L)**, and severe **(M)** phenotypes. The plants shown were 3 weeks old.
(N) and **(O)** Flowers of wild-type **(N)** and *35S:TCP3SRDX* **(O)** plants.
(P) and **(Q)** The adaxial surface of sepals of wild-type **(P)** and *35S:TCP3SRDX* **(Q)** plants. Asterisks indicate serration of the margin.
(R) and **(S)** Petals of wild-type **(R)** and *35S:TCP3SRDX* **(S)** plants.
(T) and **(U)** Stamens of wild-type **(T)** and *35S:TCP3SRDX* **(U)** plants. Arrows in **(U)** indicate outgrowths on the anther.
(V) Siliques of wild-type (bottom panel) and *35S:TCP3SRDX* (top panel) plants. st, style.
Bars = 0.5 mm in **(B)** to **(G)** and **(M)** to **(U)**, 0.1 mm in **(H)** and **(I)**, and 10 mm in **(J)** to **(L)** and **(V)**.

and severer phenotypes was lower than in the case of *35S:TCP3SRDX* plants (Figure 3). *Pro$_{TCP3}$:TCPS3RDX* plants with a mild or a moderate phenotype had leaves sepals, petals, and siliques with wavey surfaces and serrated margins (Figures 3C to 3G). These results indicate that TCP3SRDX, which is present at a concentration more similar to that of the corresponding native transcription factor, could induce the defective phenotype.

TCP3 Regulates the Expression of Boundary-Specific Genes

The *35S:TCP3SRDX* plants were morphologically similar to transgenic plants that expressed *CUC1* (*35S:CUC1*) ectopically with respect to the formation of ectopic shoots on the adaxial surface of cotyledons, wavy margins, irregular formation of

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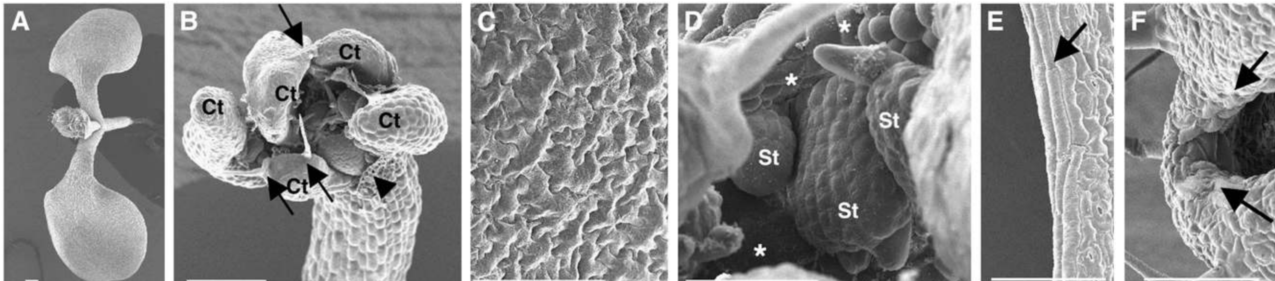

Figure 2. Scanning Electron Microscopy Analysis of Wild-Type and *35S:TCP3SRDX* Shoot Lateral Organs.

(A) and **(B)** Seedlings of a wild-type plant **(A)** and a *35S:TCP3SRDX* plant with the severe phenotype **(B)**. Ct, cotyledonary blades that had emerged from the same layer of the hypocotyl. Arrows indicate ectopic trichomes. A cotyledonary blade at the position of the arrowhead was detached to allow visualization of the interior. Bars = 200 μm.

(C) and **(D)** The adaxial surface of cotyledons of wild-type **(C)** and *35S:TCP3SRDX* **(D)** plants. Asterisks in **(D)** indicate clusters of rounded cells. St, ectopic shoots.

(E) and **(F)** Marginal regions of wild-type **(E)** and *35S:TCP3SRDX* **(F)** leaves. Arrows in **(E)** indicate rod-shaped cells that were typically observed in marginal regions and those in **(F)** indicate curling of the marginal region. Bars = 50 μm in **(C)** to **(F)**.

vasculature, and the undifferentiated rounded shape of epidermal cells (Takada et al., 2001; Hibara et al., 2003). To analyze the effects of TCP3 on the regulation of the spatial expression of *CUC1*, we introduced *TCP3SRDX* into an enhancer trap line of *CUC1*, namely, M0223 (Cary et al., 2002). In M0223 plants, we only detected the promoter activity, as displayed by the fluorescence of green fluorescent protein, in the boundary region between two cotyledons (Figure 4A). By contrast, the region in which *CUC1* was expressed had expanded broadly in cotyledons of M0223 plants that had been transformed with the *TCP3SRDX* construct (Figure 4B). Resembling results for the *CUC1* gene, the areas of expression of two other boundary-specific genes, *CUC3* and *LATERAL ORGAN BOUNDARIES* (*LOB*) (Shuai et al., 2002; Vroemen et al., 2003), as represented by signals due to β-glucuronidase (GUS) in the enhancer trap WET368 and ET22 lines, respectively, were broadly expanded in the cotyledons of the respective enhancer trap lines, when *TCP3SRDX* was expressed after it had been introduced by transformation (Figure 4C to 4F). In addition, inappropriate expression of *CUC3* was also apparent in mature leaves and sepals of the WET368 line in association with the expression of *TCP3SRDX*, while no expression of *CUC3* was evident in leaves and sepals in the absence of the *TCP3SRDX* transgene (Figures 4G to 4K). Cells in leaves in which the *CUC3* gene was ectopically expressed were often rounded (Figure 4I), suggesting that the expression of the boundary-specific genes induced an undifferentiated state in these cells. Analysis of the expression of transcripts revealed that the boundary-specific genes *CUC1*, *CUC3*, *CYP78A5*, *LOB*, *LATERAL SUPPRESSOR*, and *BLADE ON PETIOLE1* (*BOP1*) (Aida et al., 1997; Zondlo and Irish, 1999; Shuai et al., 2002; Greb et al., 2003; Vroemen et al., 2003; Ha et al., 2004) were expressed in leaves of *35S:TCP3SRDX* plants, whereas these genes were not expressed in leaves of wild-type plants (Figure 4N). Similarly, the *CUC1*, *CUC3*, and *LOB* genes were also expressed ectopically in the cotyledons of Pro_{TCP3}*:TCP3SRDX* plants (see Supplemental Figure 4 online). These results indicate that TCP3SRDX induced the ectopic expression of a variety of boundary-specific genes in a variety of organs.

In addition to its effect on boundary-specific genes, we examined the effect of TCPSRDX on the expression of class I *KNOX* genes, namely, *KNAT1*, *KNAT2*, and *SHOOT MERISTEMLESS* (*STM*), which is required for the formation of a functional meristem (Chuck et al., 1996; Ori et al., 2000; Hake et al., 2004). The promoter activity of *KNAT1* was detected only in the SAM of Pro_{KNAT1}*:GUS*

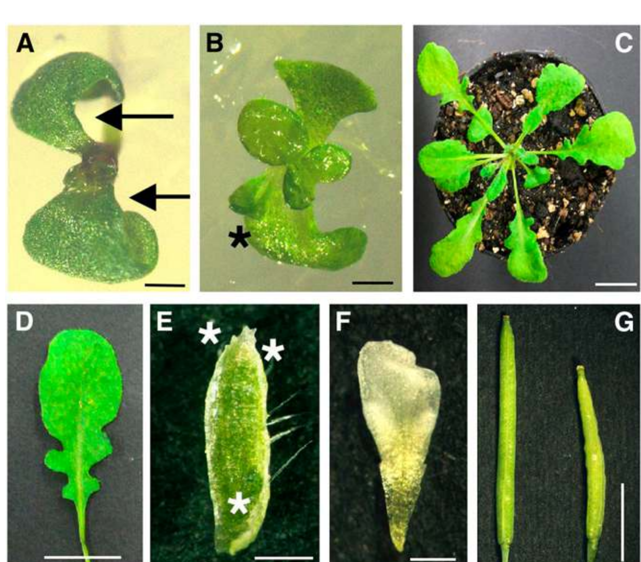

Figure 3. Phenotype of Pro_{TCP3}*:TCP3SRDX* Plants.

(A) and **(B)** Seedlings with irregular differentiation of the petiole, as indicated by arrows **(A)**, and with ectopic shoots on the cotyledon, as indicated by an asterisk **(B)**.

(C) The rosette of a Pro_{TCP3}*:TCP3SRDX* plant.

(D) A leaf of a Pro_{TCP3}*:TCP3SRDX* plant.

(E) A sepal of a Pro_{TCP3}*:TCP3SRDX* plant. Asterisks indicate serrations.

(F) A petal of a Pro_{TCP3}*:TCP3SRDX* plant.

(G) Siliques of a wild-type (left) and a Pro_{TCP3}*:TCP3SRDX* (right) plant. The silique on the right is shorter and has a crinkled surface.

Bars = 0.5 mm in **(A)**, **(B)**, **(E)**, and **(F)** and 10 mm in **(C)**, **(D)**, and **(G)**.

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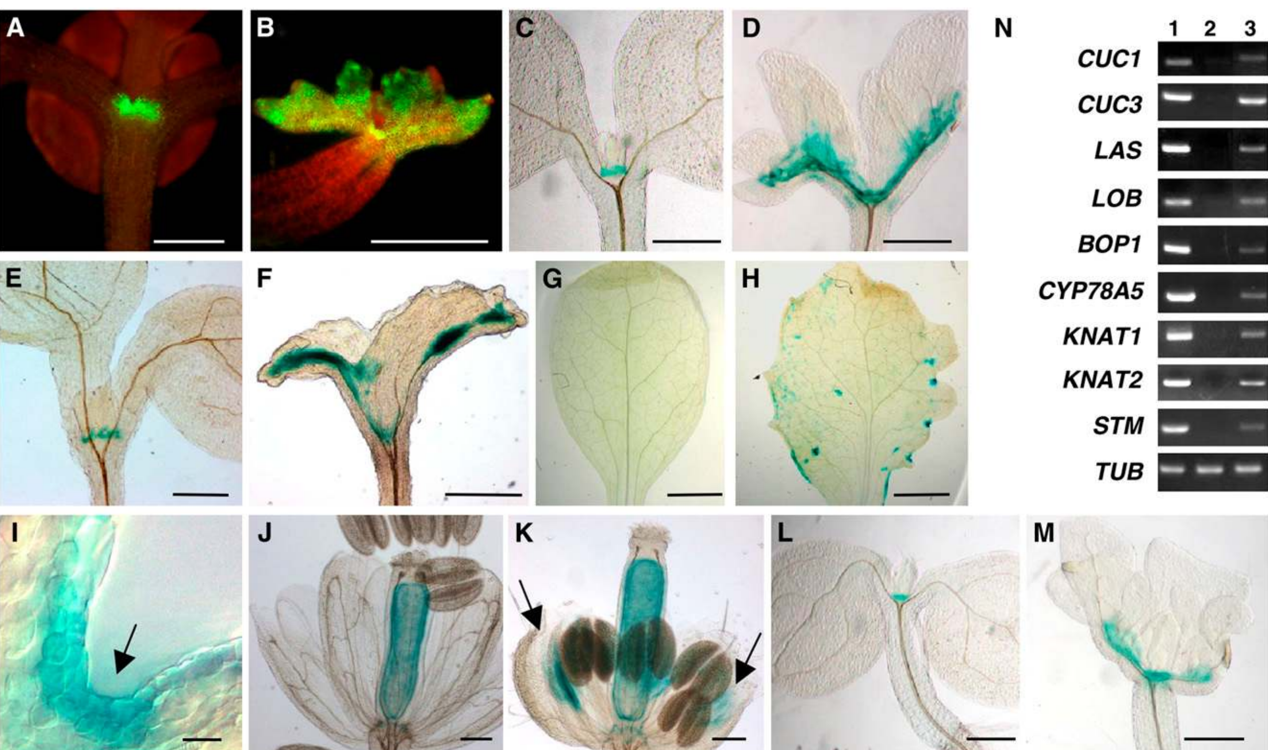

Figure 4. The Effects of *TCP3SRDX* on the Pattern of Expression of Boundary-Specific Genes and Class I *KNOX* Genes.

(A) and **(B)** Expression of *CUC1* in a seedling of the M0223 line, an enhancer trap line of *CUC1* (C24 background) **(A)**, and that in a similar seedling that expressed *TCP3SRDX* **(B)**.

(C) and **(D)** Expression of *CUC3* in a seedling of the WET368 line, an enhancer trap line of *CUC3* (Landsberg *erecta* [L*er*] background) **(C)**, and that of the same line that expressed *TCP3SRDX* **(D)**.

(E) and **(F)** Expression of *LOB* in a seedling of the ET22 line, an enhancer trap line of *LOB* (L*er* background) **(E)**, and that of the same line that expressed *TCP3SRDX* **(F)**.

(G) and **(H)** Expression of *CUC3* in rosette leaves of the WET368 line **(G)** and of the WET368 line that expressed *TCP3SRDX* **(H)**.

(I) A magnified view of curling of the leaf margin in **(H)**.

(J) and **(K)** Expression of *CUC3* in sepals of the WET368 line **(J)** and in sepals of the same line that expressed *TCP3SRDX* **(K)**. A strong GUS signal was detected between ovules, as reported previously (Vroemen et al., 2003).

(L) and **(M)** Expression of *KNAT1* in a Pro_{KNAT1}*:GUS* seedling **(L)** and in a similar seedling that expressed *TCP3SRDX* **(M)**.

(N) Analysis of the expression of boundary-specific genes and class I *KNOX* genes by RT-PCR. Lane 1, RNA isolated from wild-type seedlings as a positive control for boundary-specific genes; lane 2, RNA from wild-type leaves; and lane 3, RNA from *35S:TCP3SRDX* leaves. Expression of the gene for *Tubulin* (*TUB*) was monitored as an internal control.

Bars = 0.5 mm in **(A)** to **(F)** and **(J)** to **(M)** and 5 mm in **(H)** and **(I)**.

plants, while the region in which *KNAT1* was expressed was much more extensive in the presence of *TCP3SRDX* (Figures 4L and 4M). In addition, analysis by RT-PCR showed that *KNAT1*, *KNAT2*, and *STM* were expressed ectopically in leaves of 35S:*TCP3SRDX* plants (Figure 4N). Since the product of the *CUC1* gene is a positive regulator of the expression of class I *KNOX* genes (Takada et al., 2001; Hibara et al., 2003), it seems likely that enhanced expression of boundary-specific genes, including the *CUC1* gene, in response to TCP3SRDX induced the inappropriate expression of the class I *KNOX* genes in *35S:TCP3SRDX* plants.

The expression of *CUC* genes is negatively regulated by miR164 (Laufs et al., 2004; Mallory et al., 2004; Baker et al., 2005). To investigate whether TCP3 is involved in the accumulation of miR164, we examined levels of miR164 in 35S:*TCP3SRDX* plants. RNA gel blot analysis revealed a significant reduction in the accumulation of miR164 in 35S:*TCP3SRDX* plants (Figure 5), suggesting the involvement of TCP3 in the regulation of the accumulation of this miRNA.

Loss of CUC Activity Suppresses the Function of the Chimeric TCP3 Repressor

To confirm that the abnormal phenotype of *35S:TCP3SRDX* plants was due to the inappropriate expression of boundary-specific genes, we expressed *TCP3SRDX* in plants with a loss-of-function mutation in a boundary-specific gene. When *TCP3SRDX* was expressed on the *cuc1* mutant background, the defective phenotype of cotyledons of *35S:TCP3SRDX* plants was suppressed to a considerable extent and most of the seedlings had normal cotyledons with a flat surface and smooth margins, with

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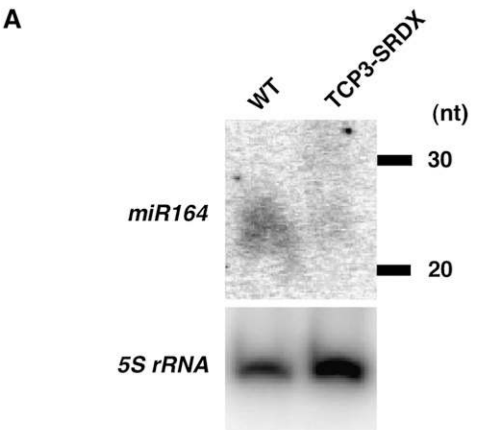

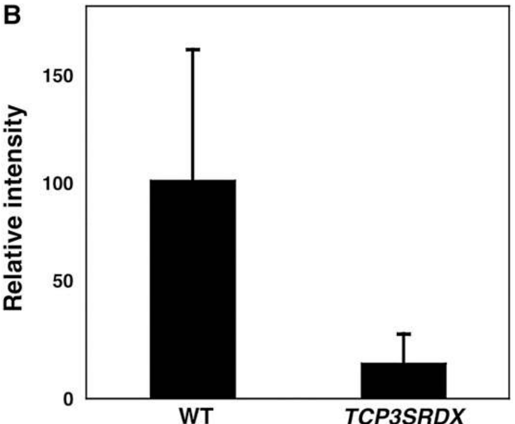

Figure 5. TCP3SRDX Suppressed the Accumulation of miR164.

(A) RNA gel blot analysis for the detection of miR164 in wild-type and *35S:TCP3SRDX* plants. *5S* rRNA was used as an internal control. nt, nucleotides.

(B) Quantitative analysis of the accumulation of miR164 in wild-type and *35S:TCP3SRDX* plants. The intensity of the signal due to miR164 is shown relative to that due to *5S* rRNA. The relative value for the wild type was set at 100. The error bar indicates the SD of results from three independent experiments.

no ectopic shoots (Figures 6A and 6B). Such recovery was observed similarly on the *cuc2* mutant background, but the frequency of recovery was lower than on the *cuc1* mutant background (Figure 6C). By contrast, no recovery was observed on the *bop1-4* mutant background (data not shown). These results indicated that the abnormal phenotype of *35S:TCP3SRDX* plants was most likely due to the inappropriate expression of the *CUC* genes.

Gain of Function of TCP3 Suppresses the Formation of Shoot Meristems

Transgenic plants that expressed *TCP3* ectopically (*35S:TCP3*) had no visible abnormalities, probably as a result of the activity of miR319/JAW (data not shown). Therefore, we expressed a mutant form of *TCP3* (*mTCP3*) in which the target site of miR319/JAW had been replaced by a nontarget sequence, without any change in the encoded amino acid sequence, as described previously in the case of *TCP2* and *TCP4* (Palatnik et al., 2003). We found that *35S:mTCP3* induced the fusion of cotyledons and defects in the formation of shoots, in addition to enhanced elongation of hypocotyls (Figures 7A to 7C). This phenotype was somewhat similar to that of the *cuc1 cuc2* double mutant. Similar fusion of cotyledons was also observed in *35S:mTCP2* and *35S:mTCP4* plants to varying degrees (Palatnik et al., 2003; see Supplemental Figure 5 online). In seedlings of these plants, the expression of *CUC1* and *CUC3* was significantly suppressed or undetectable (Figure 7D; see Supplemental Figure 5 online). Analysis by RT-PCR confirmed that the level of the expression of these *CUC* genes was clearly reduced in *35S:mTCP3* plants (data not shown). These results demonstrated that TCP3, in addition to TCP2 and TCP4, can suppress the expression of *CUC* genes.

Redundant Functions of the Members of the TCP Family

As compared with transgenic plants that expressed TCP3SRDX, we found that *tcp3-1* plants, namely, the *TCP3* T-DNA–tagged homozygous line (CS855978), and transgenic plants that expressed the double-stranded RNA for RNA interference (RNAi) of the *TCP3* gene had basically normal cotyledons (see Supplemental Figure 6 online). By contrast, ectopic expression of a genomic DNA fragment that encoded miR319/JAW, which should cleave transcripts of the *TCP2*, *TCP3*, *TCP4*, *TCP10*, and *TCP24* genes, resulted in cotyledons that resembled those of *35S:TCP3SRDX* plants with the mild phenotype (see Supplemental Figure 6 online), in addition to an effect on leaf phenotype,

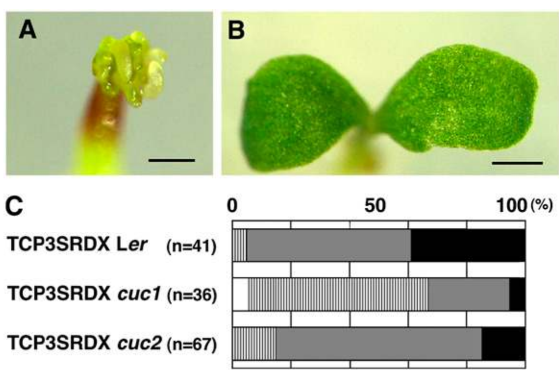

Figure 6. Mutations in *CUC* Genes Suppressed the Activity of TCP3SRDX.

(A) A *35S:TCP3SRDX* L*er* seedling with abnormal cotyledons and ectopic shoots.

(B) A *35S:TCP3SRDX cuc1* seedling, showing cotyledons with normal morphology. Bars = 0.5 mm in **(A)** and **(B)**.

(C) Schematic representation of the frequency of reversal of the abnormal phenotype of *35S:TCP3SRDX* seedlings by mutations in *CUC* genes. Phenotypic severity was classified as indicated in Figures 1C to 1E. Open box, similar to the wild type; striped box, mild phenotype; gray box, moderate phenotype; and closed box, severe phenotype. The number of seedlings examined is given in parenthesis in each case. The data are given as percentages. The background of the *cuc* mutants was the L*er* ecotype.

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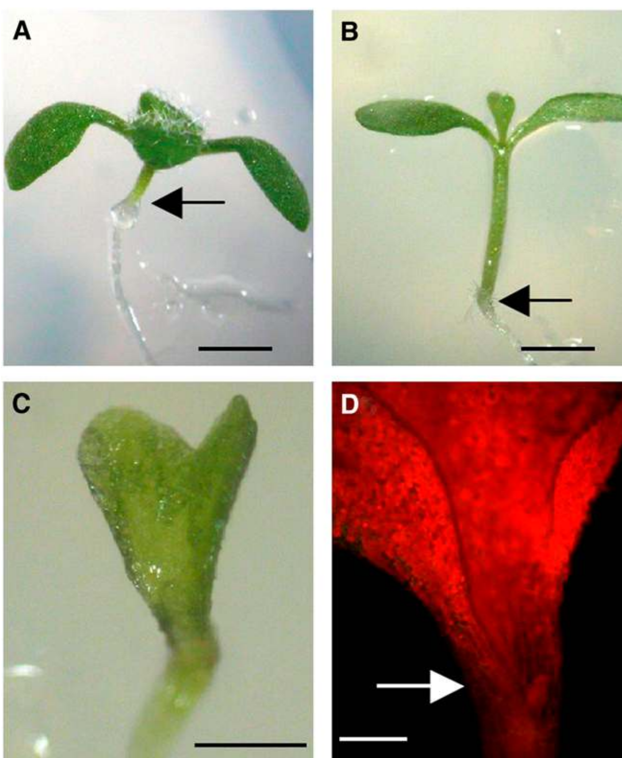

Figure 7. Gain of Function of TCP3 Activity Inhibited Formation of Shoots.

(A) and **(B)** Side views of a wild-type seedling **(A)** and of a *35S:mTCP3* seedling that had a longer hypocotyl **(B)**. Arrows indicate the junction of the hypocotyl and the main root.

(C) Fused cotyledons lacking shoots in a *35S:mTCP3* seedling.

(D) Expression of the *CUC1* gene in the M0223 line that expressed *mTCP3*. Expression of *CUC1* was suppressed in the presumptive boundary region of the M0223 seedling, as indicated by an arrow.

Bars = 0.5 mm in **(A)** to **(C)** and 0.1 mm in **(D)**.

as reported previously (Palatnik et al., 2003). These analyses suggest that suppression of the expression of five *TCP* genes might be required for induction of cotyledons with the abnormal phenotype because of the functional redundancy of *TCP* genes.

We produced seven lines of transgenic *Arabidopsis* plants that expressed individual chimeric repressors derived from each of seven other TCPs in the CYC/TB subfamily, namely, TCP2, TCP4, TCP5, TCP10, TCP13, TCP17, and TCP24. We found that all seven chimeric repressors induced phenotypes similar to that induced by TCP3SRDX, although the severity of the phenotypes differed even when gene expression was driven by the CaMV 35S promoter (see Supplemental Figures 6A and 6B online). In these transgenic plants, we confirmed that the boundary-specific genes were expressed ectopically in cotyledons and leaves, as they had been also in *35S:TCP3SRDX* plants (see Supplemental Figures 7A and 7B online). Moreover, expression of *TCP10SRDX* driven by its homologous promoter (*Pro_{TCP10}:TCP10SRDX*) induced defects in cotyledons and the ectopic expression of boundary-specific genes similar to those observed when transcription was driven by the CaMV 35S promoter (see Supplemental Figure 8 online). These observations suggested that the TCP transcription factors examined in this study have similar molecular functions and are possible regulators of the expression of boundary-specific genes.

The Patterns of Expression of the *TCP* Genes

In situ hybridization revealed that the *TCP3* transcript was localized in the distal and middle regions of cotyledons of torpedo, bending cotyledon, and mature embryos, but no signals were detected in the presumptive SAM and the boundary region during embryogenesis (Figures 8A to 8E). Resembling the *TCP3* transcript, the *TCP10* transcript was also detected in regions of the developing embryo except the region of the presumptive SAM and the boundary region (Figures 8F to 8I). In contrast with the *TCP3* transcript, however, the *TCP10* transcript was detectable in the cotyledons of heart-stage embryos (Figure 8F). Since expression of the *TCP4* gene is detectable in embryonic cotyledons (Palatnik et al., 2003), these three *TCP* genes have similar patterns of expression in cotyledons.

We also examined the patterns of expression of *TCP* genes using a GUS reporter gene that was fused with the 5′-upstream region of each respective *TCP* gene (*Pro_{TCP}:GUS*). The promoter activity of the *TCP3* gene, as represented by the GUS activity due to the *Pro_{TCP3}:GUS* gene, was strong in the vascular regions of the cotyledons and hypocotyl in torpedo and bending cotyledon embryos, as well as in developing seedlings (Figures 8J to 8N). The difference between the patterns of expression of *TCP3* during early embryogenesis obtained with *Pro_{TCP3}:GUS* and by in situ analysis might have been due to posttranscriptional suppression by the miR319/JAW-mediated cleavage of the *TCP3* transcript or to transcriptional regulation via sequences beyond the promoter region used in this study. The promoters of the *TCP2*, *TCP4*, *TCP5*, *TCP10*, *TCP13*, *TCP17*, and *TCP24* genes were active in cotyledons and, in particular, in the vascular region, as was the case for the promoter of the *TCP3* gene, even though each gene had a somewhat different pattern of expression (see Supplemental Figure 9 online). Analysis by RT-PCR confirmed that these eight *TCP* genes are expressed differentially in various organs in an overlapping manner, although no signal for *TCP5* was detected in roots (Figure 8O).

DISCUSSION

In this study, we found that the expression of a chimeric TCP3 repressor induced the ectopic expression of boundary-specific genes, with the resultant formation of ectopic shoots, while overexpression of *mTCP3* suppressed the expression of such genes, with resultant inhibition of the formation of shoots and of the separation of cotyledons. Our results demonstrate that TCP3 plays a pivotal role in the control of morphogenesis of shoot lateral organs via the negative control of the expression of boundary-specific genes, namely, the *CUC* genes, which control the initiation of the shoot meristem and the morphogenesis of organ boundaries (Aida et al., 1997). Given that TCP3 has transactivational activity, we can postulate that TCP3 might activate some unidentified factors that suppress the transcription of the *CUC* genes. The proposed function of TCP3 is consistent with the

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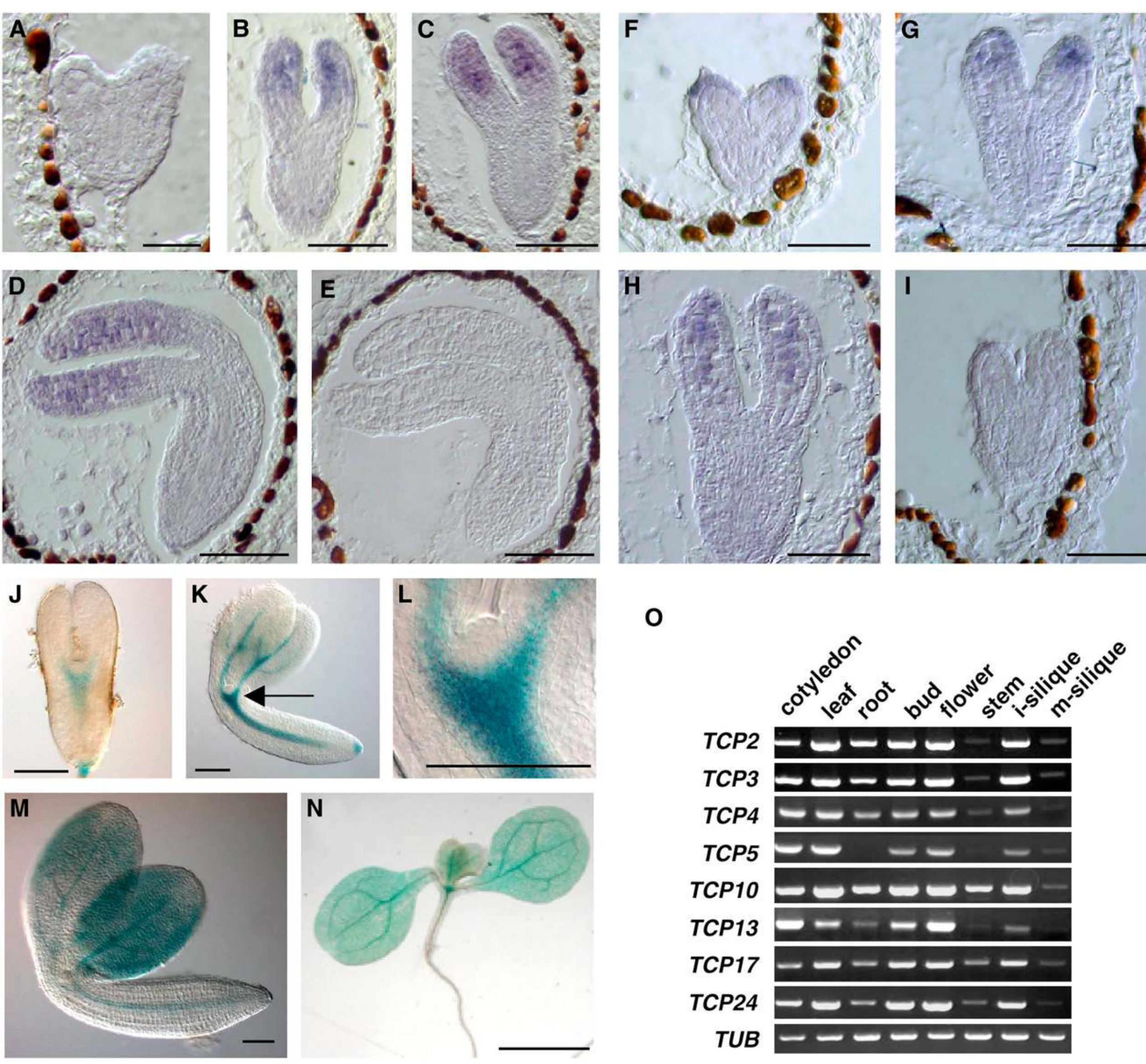

Figure 8. Expression of *TCP* Genes.

(A) to **(E)** Detection by in situ hybridization of transcripts of the *TCP3* gene. The signal due to *TCP3* transcripts was absent from heart-shaped embryos **(A)** but was present in cotyledons of torpedo **(B)**, bending cotyledon **(C)**, and mature **(D)** embryos. No signal was detected when the sense probe of the *TCP3* gene was used **(E)**.

(F) to **(I)** Detection by in situ hybridization of transcripts of the *TCP10* gene. The signal due to *TCP10* transcripts was detected in cotyledons of heart-shaped **(F)**, torpedo **(G)**, and bending cotyledon **(H)** embryos. No signal was observed when the sense probe for the *TCP10* gene was used **(I)**.

(J) to **(N)** Expression of the Pro_{TCP3}*:GUS* reporter gene in embryos at the torpedo stage **(J)** and the bending cotyledon stage **(K)**. In **(L)**, a magnified view of the SAM region is shown that is indicated by an arrow in **(K)**. A mature embryo **(M)** and a seedling **(N)** are also shown.

(O) Analysis of the expression of eight *TCP* genes in various organs by RT-PCR. Expression of the gene for *Tubulin* (*TUB*) was monitored as an internal control. i-silique, immature silique; m-silique, mature silique.

Bars = 50 μm in **(A)** to **(M)** and 1 mm in **(N)**.

finding that the pattern of expression of the *TCP3* gene does not overlap with that of *CUC* genes.

The expression of the *CUC* genes is regulated at the transcriptional level but is also regulated negatively by miR164 (Laufs et al., 2004; Mallory et al., 2004; Baker et al., 2005). miR164 is encoded by three genes, *miR164A*, *miR164B*, and *miR164C*, and it accumulates in various tissues, which include seedlings, leaves, and floral organs (Laufs et al., 2004; Mallory et al., 2004; Baker et al., 2005). The suppression of expression of miR164 in a T-DNA–tagged line for the *miR164A* gene deepened the serration of leaf margin (Nikovics et al., 2006), and this phenotype was somewhat similar to that of *35S:TCP3SRDX* plants. Thus, control of the

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accumulation of miR164 might be important for appropriate development of leaves. We showed in this report that the level of miR164 was significantly reduced in *35S:TCP3SRDX* plants (Figure 5). It is possible that suppression of the expression of the *miR164* gene is also involved in the ectopic expression of *CUC* genes in *35S:TCP3SRDX* plants. However, considering that mutation of the *miR164A* gene did not induce ectopic shoots, we postulate that TCP3 might control the expression of the *CUC* genes at both the transcriptional and posttranscriptional levels.

Our results suggest that elimination from cotyledons of the activities of proteins encoded by the *CUC* and other boundary-specific genes might be required for the normal development of shoot lateral organs. Since the *CUC* genes and several boundary-specific genes appear to control morphogenesis at boundaries and the formation of shoot meristems (Aida and Tasaka, 2006a, 2006b), inappropriate expression of these genes might prevent the specific differentiation of cells that is necessary for the formation of smooth surfaces and margins of organs. Indeed, ectopic expression of the *CUC* genes and other boundary-specific genes has been reported to disturb the normal development of the respective transgenic plants (Zondlo and Irish, 1999; Takada et al., 2001; Shuai et al., 2002; Hibara et al., 2003; Laufs et al., 2004). The abnormal distribution of or response to auxin induces the inappropriate expression of *CUC* genes in cotyledons and lateral organ primordia, preventing organ development (Vernoux et al., 2000; Furutani et al., 2004; Treml et al., 2005). In addition, the abnormal radial information generated by inappropriate expression of *PINHEAD* in the peripheral region of an embryo induces ectopic expression of *CUC2* and the formation of shoot meristems on cotyledons (Newman et al., 2002). Thus, the spatial regulation of the expression of the *CUC* genes and other boundary-specific genes might be a critical determinant in the control of morphogenesis of shoot lateral organs. TCPs appear to be novel factors that are involved in this regulation.

By contrast, it appears that initiation of the formation of shoot meristems requires suppression of the activities of TCPs in the boundary region and the SAM. The accumulation of transcripts of the *TCP3*, *TCP4*, and *TCP10* genes and the activities of the respective promoters were not evident either in the SAM or at cotyledonary boundaries (Figure 8; Palatnik et al., 2003). In addition, we found that ectopic expression of *mTCP3*, in which the target site of miR319/JAW had been mutated, inhibited formation of shoots, probably as a result of the expression of *TCP3* in the boundary region. Consistent with these observations, the precursor to miR319/JAW is abundant in the shoot apex, which includes the SAM (Palatnik et al., 2003). Thus, the miR319/JAW-dependent cleavage of *TCP* transcripts might be required for the formation of the shoot meristem. Our results suggest that suppression of the activities of TCPs in the boundary region might be controlled at both the transcriptional and the posttranscriptional level.

We showed that eight TCPs have similar molecular functions by examining the effects of the chimeric repressors derived from each one, and we found that the corresponding *TCP* genes were expressed in an overlapping manner in various tissues. The *TCP3*, *TCP4*, and *TCP10* genes have similar patterns of expression in cotyledons during embryogenesis, and seven of the eight *TCP* genes are expressed during the early development of flowers (Figure 8; Palatnik et al., 2003; Wellmer et al., 2006). In addition, the functional redundancy of these TCPs was apparent from the observation that neither T-DNA–tagged lines nor RNAi for *TCP3* induced an informative phenotype in *Arabidopsis.* Although *35S:miR319/JAW* plants have mildly abnormal cotyledons, the functions of the *TCP5*, *TCP13*, and *TCP17* genes, whose transcripts lack a target sequence for miR319/JAW, might prevent formation of more severely abnormal cotyledons, with, for example, ectopic shoots. These observations suggest that the TCP transcription factors of *Arabidopsis* act redundantly to regulate the spatial expression of boundary-specific genes and to control the morphogenesis of shoot lateral organs. *Arabidopsis* appears to have a greater number of redundant *TCP* genes than does *Antirrhinum* because disruption of the *CIN* gene alone is sufficient to induce abnormal leaves in *Antirrhinum* (Nath et al., 2003).

Each *TCP* gene had a somewhat different pattern of expression, and each chimeric TCP repressor induced an abnormal phenotype with a different degree of severity, even when transcription of its gene was driven by the CaMV 35S promoter. The chimeric repressors derived from TCP3, TCP4, TCP5, and TCP10 induced the severe phenotype at higher frequency, perhaps because of higher activities of DNA binding and transactivation. In addition, the activities of the promoter regions of the *TCP3*, *TCP4*, *TCP5*, and *TCP10* genes used in this study were more prominent in cotyledons. These genes might make a larger contribution to the development of cotyledons than do other *TCP* genes. Thus, the TCP transcription factors examined in this study appear to function similarly as regulators of the expression of boundary-specific genes, but the spatial activity of each TCP seems to be regulated differently.

Because of the close relationships among TCPs, in terms of phylogeny, and their functional similarities, we have grouped these eight TCPs as a subfamily of CIN-like TCPs. *TCP* genes are conserved in monocots, as well as in dicots, and there are 23 *TCP* genes in the rice (*Oryza sativa*) genome, just as there are in the *Arabidopsis* genome. Ten of these rice genes, which include genes for class II PCFs (Kosugi and Ohashi, 2002), can be considered to be *CIN*-like *TCP* genes. We found that five of the *CIN*-like *TCP* genes in rice have a target sequence for miR319/JAW in addition to those mentioned in a report by Palatnik et al. (2003). Thus, it is likely that not only the family of *TCP* genes but also the mechanism for regulation of the expression of genes for TCPs by the *miR319/JAW* gene evolved prior to the divergence of monocots and dicots. It has been suggested that *CUC* genes might be involved in the formation of the SAM in monocots (Zimmermann and Werr, 2005). Thus, the acquisition of a mechanism for regulation of the spatial activities of *CUCs* by TCPs might have been a key event in the evolution of plants.

In this study, we showed that TCP transcription factors are critical for the morphogenesis of shoot lateral organs. Extending the previous reports that TCPs are involved in the control of the development of leaves (Nath et al., 2003; Palatnik et al., 2003), we showed that TCPs are involved in the formation of the shoot meristem and the development of shoot lateral organs. When TCPs fail to suppress the expression of the *CUC* genes, ectopic expression of *CUC* genes results in the generation of multiple

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shoot meristems and inhibition of organ growth. Plant cells have extraordinary totipotency and have the ability to generate new organs continuously from shoot meristems and to form shoot meristems at organ boundaries throughout their life cycle (Weigel and Jurgens, 2002; Willemsen and Scheres, 2004; Schmitz and Theres, 2005; Aida and Tasaka, 2006a, 2006b). This process requires the coordination of the functions of the shoot meristem with those of the differentiating cells of a given organ. Thus, the TCP-dependent regulation of the expression of *CUC* genes, which controls morphogenesis of the shoot lateral organs and regulates formation of the shoot meristem, is of critical importance to plant development.

METHODS

Construction of Plasmids

The protein-coding regions of *TCP* genes were amplified from genomic DNA or from cDNAs provided by the RIKEN Bio Resource Center (BRC; Seki et al., 1998, 2002) with appropriate primers, as shown in Supplemental Table 1 online. The chimeric *35S:TCPSRDX* gene was constructed as described previously (Mitsuda et al., 2006). The *mSRDX*, *mTCP2*, *mTCP3*, and *mTCP4* genes were generated by site-directed mutagenesis with appropriate primers. The *GUS* reporter genes under the control of the promoters of individual *TCP* genes were constructed using 5′-upstream regions relative to sites of initiation of translation of the respective *TCP* genes (*TCP2*, 2978 bp; *TCP3*, 2593 bp; *TCP4*, 3027 bp; *TCP5*, 2879 bp; *TCP10*, 1054 bp; *TCP13*, 2863 bp; *TCP17*, 2276 bp; and *TCP24*, 2776 bp). The *GUS* genes of Pro_{TCP3}*:GUS* and Pro_{TCP10}*:GUS* were replaced by *TCP3SRDX* and *TCP10SRDX* for the construction of Pro_{TCP3}*:TCP3SRDX* and Pro_{TCP10}*:TCP10SRDX*, respectively. The construct for RNAi of *TCP3* was generated from the 458-bp region at the 3′ end of the gene (nucleotides 715 to 1173) and pHELLSGATE8 (Wesley et al., 2001).

Plant Materials and Transformation

Arabidopsis thaliana ecotype Col-0 was used throughout this study unless otherwise indicated. Growth conditions and the strategy for transformation of *Arabidopsis* were described previously (Mitsuda et al., 2006). The exogenous expression of each transgene in transgenic plants was confirmed by RT-PCR with appropriate primers (see Supplemental Figure 10 and Supplemental Table 1 online). The *tcp3-1* line had a T-DNA tag in the protein-coding region of the *TCP3* gene at a site 261 bp from the site of initiation of translation.

Light and Scanning Electron Microscopy

Analysis of promoter activities using the GUS reporter gene was performed with T1 or T2 transgenic lines as described previously (Mitsuda et al., 2005). Light microscopy and fluorescence microscopy for detection of green fluorescent protein were performed with the Axioskop2 plus system (Carl Zeiss). Plant tissues were rendered transparent for the observations of vasculature as described previously (Aida et al., 1997). For scanning electron microscopy, samples were prepared and analyzed as described previously (Mitsuda et al., 2005).

Isolation of RNA and RT-PCR

Total RNA was isolated from tissues with Trizol as described previously (Fujimoto et al., 2000). For analysis by RT-PCR, aliquots of 50 ng of total RNA were subjected to first-strand cDNA synthesis (Hiratsu et al., 2003). PCR was performed with gene-specific primers (see Supplemental Table 1 online) for 30 to 39 cycles.

Small RNA was prepared with a *mir*Vana miRNA isolation kit (Ambion) from 3-week-old plants. For detection of miR164, aliquots of 40 μg of small RNA were fractionated on a 15% polyacrylamide gel that contained 7 M urea, blotted onto a nylon membrane, and allowed to hybridize to the ^{32}P-labeled nucleotide probe in ULTRAhyb-Oligo solution (Ambion). The synthetic RNA corresponding to the sense strand of *miR164* was used as a positive control for hybridization. The intensity of signals was quantified with ImageQuant (Molecular Dynamics).

In Situ Hybridization

Preparation of samples and in situ hybridization were performed as described previously (Furutani et al., 2004). DNA fragments corresponding to positions 48 to 1176 from the site of initiation of translation of *TCP3* and to the full-length coding sequence of *TCP10* were used as templates for probes, respectively.

Assays of Transient Gene Expression

The coding sequences of *TCP3*, *TCP3SRDX*, and *TCP3mSRDX* were fused separately to that for GAL4DB (Ohta et al., 2000), and assays of transient gene expression were performed as described previously (Fujimoto et al., 2000). We used 0.8 μg of reporter plasmid and 0.6 μg of effector plasmid for each bombardment. For normalization of the activity of the reporter gene, we used 0.8 μg of a reference plasmid, pPTRL (Fujimoto et al., 2000). After bombardment, samples were incubated for 16 h in darkness, and then luciferase activity was quantified.

Accession Numbers

The Arabidopsis Genome Initiative (http://www.arabidopsis.org) identifiers for the genes and gene products were designated as follows: TCP2, At4g18390; TCP3, At1g53230; TCP4, At3g15030; TCP5, At5g60970; TCP10, At2g31070; TCP13, At3g02150; TCP17, At5g08070; and TCP24, At1g30210.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table 1. Primers Used in This Study.

Supplemental Figure 1. Phylogeny of TCPs in the CYC/TB Subfamily.

Supplemental Figure 2. Inhibition of Elongation of the Main Root by the Chimeric TCP Repressor.

Supplemental Figure 3. Transient Expression Assay for TCP3.

Supplemental Figure 4. Expression of Boundary-Specific Genes in Pro_{TCP3}*:TCP3SRDX* Plants.

Supplemental Figure 5. Phenotype Induced by the Ectopic Expression of the *mTCP* Gene.

Supplemental Figure 6. Phenotype Induced by Suppression of the Expression of the *TCP* Gene.

Supplemental Figure 7. Phenotypes Induced by Seven Different Chimeric TCP Repressors.

Supplemental Figure 8. Phenotype of Pro_{TCP10}*:TCP10SRDX* Plants.

Supplemental Figure 9. Analysis of the Promoter Activities of *TCP* Genes in Young Seedlings.

Supplemental Figure 10. Expression of the *TCP3SRDX* Gene in *35S:TCP3SRDX* Plants.

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